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(54) Title: TRANSGENIC ANIMALS HAVING A DEFECTIVE THYROID HORMONE RECEPTOR BETA GENE

(57) Abstract

The invention provides a transgenic mammal which is heterozygous or homozygous for an at least partially defective thyroid hormone receptor β gene, cells derived from the mammal and methods for the use of the mammal and the cells.

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TRANSGENIC ANIMALS HAVING A DEFECTIVE THYROID HORMONE RECEPTOR BETA GENE

This application relates to transgenic animals, particularly mice, and tissues and cell lines thereof that in a homozygous form lack the gene for thyroid hormone receptor β (TRβ). The mice, tissues and cell lines of the invention may be used in the testing for pharmaceutical or clinical purposes of substances such as thyroid hormones T_3 and T_4 and possible antagonists and agonists thereof.

The thyroid hormones tri-iodothyronine (T_3) and thyroxine (T_4) have a very wide range of effects. In adult mammals they influence nearly all organs, the metabolism of nutrients, basal metabolic rate and oxygen consumption. In humans, the deficiency or excess of circulating thyroid hormones results in the well characterised syndromes hypo- and hyperthyroidism.

The thyroid hormones are essential for the normal development of the central nervous system particularly in the foetal and neonatal stages [1-6]. Deficiencies in the action of thyroid hormones lead to hypothyroidism that can be due to either acquired or congenital disorders. Some of the congenital causes of hypothyroidism are embryopathies as absence, hypoplasia, or ectopic localization of the thyroid gland; enzymatic disorders; deficient hormone synthesis and receptor disorders (Generalized Thyroid Hormone Syndrome (GRTS)). Unless treated, congenital hypothyroidism leads to irreversible mental retardation and short stature (dwarfism). Other symptoms include neurological dysfunctions such as poor coordination and balance, abnormal fine motor movements, speech problems, spasticity, tremor and hyperactive

deep tendon reflexes. In addition basal metabolic rate, gluconeogenesis, lipogenesis and cardiac output are decreased. Hypothyroidism in adults leads to symptoms similar to those described above, except for the mental retardation. However, adult patients are easily treated with hormone therapy.

In contrast to congenital hypothyroidism, hyperthyroidism is more common in adults. In general, the symptoms are the reverse: increased metabolism, lower serum cholesterol levels, hyperactivity and tachycardia are hallmarks of elevated T3/T4 levels [25].

Thyroid hormones act through thyroid hormone receptors (TRs) which belong to the superfamily of steroid hormone receptors. TRs are ligand dependent transcription factors which regulate the transcription of their target genes through responsive elements in the DNA. In vertebrates there are a variety of TRs[7-13] (Fig. A) derived from TRα and TRβ genes, which are located at the 17th and 3rd chromosomes respectively in humans. There is considerable homology between the TRα and TRβ proteins and between the receptors in different species, such as rat, mouse, and human. The α-gene encodes the subtypes α1 and α2. The α2 subtype is not a functional receptor in the sense that it lacks T_3/T_4 hormone binding capability. The β-gene encodes the subtypes β1 and β2. The latter has so far been identified only at the messenger RNA level. The physiological significance of these different proteins has not yet been clarified. Different amino- and carboxy-termini for the TR variants suggest different trans-activating properties for TRα and Trβ. In addition, the differential expression during brain development suggest different roles for the TR variants during development [14-16].

The mechanism of T_3 action via its receptor is quite complex due to the presence of multiple TRs [17-20]. The TRα locus encodes in addition to the TRα gene another receptor denoted as Rev-α. Rev-α arises by transcription of the opposite strand of TRα gene and overlaps the α2 region at the 3' end (Fig. B). Furthermore, there are TRα2 and TRα3 variants; the protein sequence of the latter is identical to that of TRα2 with the exception that it lacks the first 42 amino acids of the carboxy terminus (Fig. C).

In humans, the GRTS has been related to TRβ gene disorders. No clinical syndromes have yet been associated to TRα gene mutations suggesting that the TRα gene is either dispensable or essential for life. It is equally unclear as to which of the two thyroid hormone receptors the actions of thyroid hormones can be ascribed in hypo- and hyperthyroidism. If the individual functions in hormone action of the receptors could be identified, agonists or agonists that are specific for either of the receptors could be used for treatment of specific target tissues without adversely affecting other tissues.

Treatment of many diseases associated with thyroid hormone function cannot be done today since administration of increased doses of the hormone to achieve a desired effect in a given tissue, leads to adverse effects in another. The effects of thyroid hormones are mediated by two different receptors that are coexpressed in some tissues, whereas other tissues express only one of them. It should therefore be possible to design agonists and antagonists that are specific for each of the receptors and that can mediate a desired activation or repression of receptor function.

In order to allow testing of such components we have disrupted the TRβ gene in the mouse genome, and bred such animals to homozygosity. These animals can grow to at least sexual maturity, and are therefore suitable tools for identifying the action of agonists and antagonists of TRβ.

According to one aspect of the invention there is provided a transgenic mammal which is heterozygous for an at least partially defective thyroid hormone receptor β gene. The defective gene may be inactivated for example by an insertion, deletion, substitution or inversion or any other suitable genetic manipulation.

Preferably, the mammal is a rodent, more preferably a mouse.

One heterozygous transgenic mammal in accordance with the invention may be bred with another such heterozygous transgenic mammal to produce a mammal which is homozygous for a defective thyroid hormone receptor β gene. Thus according to another aspect of the invention there is provided a transgenic mammal which is homozygous for an at least partially defective β thyroid hormone receptor β gene.

The invention also provides cells derived from the animal of the invention which are heterozygous or homozygous for a defective thyroid hormone receptor β.

According to another aspect of the invention there is provided a method of producing a transgenic animal in accordance with the invention the method comprising :

1) preparing a gene encoding an at least partially defective thyroid hormone receptor β as described above;

2) introducing that gene into suitable carrier cells;

3) inserting those carrier cells into an embryo; and

4) replacing the embryo into a mother, and allowing the embryo to develop to full term.

According to a further aspect of the invention there is provided a method of testing the agonist/antagonist properties of a compound in relation to the thyroid hormone receptor, the method comprising:

contacting a transgenic animal in accordance with the invention with the compound and monitoring subsequent development of the animal.

Alternatively, the method may involve using cells or tissues derived from the transgenic animal.

The transgenic mammal of the invention is suitable for testing the effects of agonists and antagonists of thyroid hormone action, in particular those that discriminate between TRα and TRβ. In particular, the transgenic mammal of the invention or cells or tissues derived therefrom can be used to study the following:

1. Administration of excess thyroid hormones decreases high serum cholesterol levels. However, an adverse side effect is that cardiac output also increases which can lead to arrythmia. If these two functions of thyroid hormones are mediated by distinct

receptors, a proper administration of receptor specific agonists or antagonists would lead to the desired decrease in serum cholesterol while leaving cardiac function normal.

2. Hypo- and hyperthyroidism adversely affect bone structure. The use of receptor-specific thyroid hormone antagonists or agonists for treatment of e.g hypercholesterolemia or other diseases must therefore include a test for their influence on bone synthesis and turnover.

3. Regulation of heart functions such as pulse, arrythmia, or myocardiac muscle can be targeted by the use of receptor specific thyroid hormone antagonists or agonists.

4. Many organs or tissues produce hormones in a thyroid hormone dependent manner. Such tissues include the hypophysis (producing growth hormone, prolactin, thyroid stimulating hormone, luteinizing hormone), the hypothalamus (thyrotropin releasing hormone, oxytocin), peripheral tissues (insulin growth factor I). The effect of receptor specific thyroid hormone antagonists or agonists on such endocrine systems can be determined with the mammals of the present invention.

5. Basal metabolic rate, gluconeogenesis, lipogenesis, lipolysis and thermogenesis are increased in hyperthyroidism and decreased during hypothyroidism. The effect of receptor specific thyroid hormone antagonists or agonists on such metabolic processes can be determined with the mammal of the present invention.

6. Toxic effects of agonists and antagonists on normal and abnormal physiological metabolic processes.

7. Effects on brain or other neuronal function (hearing, peripheral nervous system), as well as effects on embryonal and foetal development of receptor specific thyroid hormone antagonists or agonists on such endocrine systems can be determined with the transgenic mammal of the present invention.

8. Effects on increasing or decreasing body growth in patients with growth disorders.

9. A large number of genes or gene products are known to be regulated by thyroid hormones. The effects of agonists and antagonists of such systems before clinical trials can commence.

10. Effect on haemopoiesis. Hypothyroid patients are usually anaemic.

11. Treatment of patients that have defective TRα receptor genes. As mentioned above, no patients with mutant TRα genes have been found, whereas genetic defects in more than 250 patients with defective TRβ genes have been identified. The latter patients were first clinically identified due to their inappropriate levels of thyroid hormones and other thyroid hormone regulated hormones such as TSH. It is therefore possible that diseases due to defects in the TRα gene have remained undetected because the patients have normal T_3/T_4 and TSH levels and their symptoms therefore would not

be easily associated with a receptor dysfunction. The TRβ deficient mammals of the present invention allow the identification of such disease, symptoms, and their cure with suitable agonists.

Mammals in accordance with the invention and their production will now be described by way of example only with reference to the further accompanying drawings Figures 1-2 in which:

Fig. 1 illustrates disruption of the TRβ gene by homologous recombination; and

Fig. 2 illustrates an RT-PCR analysis of products of the wild type and mutant alleles of the TRβ gene.

Example 1

Generation of mutant mouse with deleted thyroid hormone receptor β gene.

EXPERIMENTAL PROCEDURES

Targeting vector

A chick TRβ cDNA insert was used to screen a bacteriophage lambda library of genomic DNA of a 129sv strain mouse (Stratagene) to obtain overlapping clones that encompassed the entire coding domain of the TRβ gene. Fig. 1A is a schematic representation of the TRβ1 protein showing the central DNA binding domain (filled in black) and C-terminal T3-binding domain. Fig. 1B top line, illustrates the structure of the central region of the gene containing the first three coding exons that are common for both TRβ1 and TRβ2 (here numbered 3 to

5). The middle line illustrates the targeting vector contained 3 kbp and 4 kbp respectively of 5′ and 3′ homologous flanking DNA and carried a 3 kbp deletion including part of exon number 3. The bottom line shows the structure of the mutant allele generated by homologous recombination 5′, nco and 3′ probes used in Southern blot analyses are shown as well as the band sizes predicted to be detected with the 3′ probe following digestion with Barn HI and Eag I: the wild type band size being 19 kbp whereas the mutant band is 10 kbp. Restriction enzyme sites are indicated where relevant. X, Xba I; B, Bam HI; K, Kpn I; E, Eag I. The exon structure was confirmed by DNA sequencing of plasmid sub-clones. The targeting vector (Figure 1B) contained from 5′ to 3′: a TK gene fragment from pMCI-HSV TK, a 3 kbp fragment of TRβ genomic DNA extending to a Kpn 1 site in the coding exon number 3, a neomycin resistance gene from pgkneobpA, a 4 kbp Xba-I-Hind III genomic fragment containing the TRβ exons 4 and 5. The construct was linearized at the 5′ end of the TK gene by Bam HI digestion prior to electroporation.

Electroporation and selection of ES cells

W9.5 male ES cells derived from 129/sv mice were grown on feeder layers of G418-resistant primary mouse embryo fibroblasts (PMEFs) in dishes that had been treated with 0.1% gelatin: PMEFs were mitotically inactivated by gamma-irradiation. W9.5 cells were cultured in Dulbecco's Modified Eagle medium (Specialty Media) supplemented with 15% defined fetal bovine serum (Hyclone), 1000 U/ml of recombinant LIF (Gibco), L-glutamine, non-essential amino acids, β-mercaptoethanol and antibodies as described [26] 3 x 10^7 W9.5 cells at passage 12 were resuspended in 0.8 ml PBS containing 25μg of linearized targeting vector DNA for electroporation using a Bio-Rad Gene Pulser (500μF, 250V). Cells were then plated onto

60mm dishes. The next day the medium was replaced with medium containing 350 μg/ml G418 (dry weight, Gibco) and on day two, 2μM ganciclovir (a gift of Syntex Corp. Palo Alto, CA) was added. The medium was replaced each day and on day 8, colonies were picked and transferred into 48 well dishes. After 4-5 days growth in 48-well plates, each clone was trypsinized and 9/10 of the suspension volume removed for DNA preparation. To the remaining volume, fresh medium and PMEFs were added. Clones identified as positive for homologous recombination were expanded and stocks frozen. The chromosome content of positive clones was determined by growth on microscope chamber slides for analysis *in situ*.

Southern blot hybridization analysis of ES cell clones and genotype determination

ES cells colonies were screened for homologous recombinants in pools of six. Cell pellets were lysed at 55°C overnight and DNA was prepared and digested overnight with Bam HI and Eag I, then analyzed on 0.7% agarose gels. DNA was transferred to Duralose-UV membrane and hybridized using Quickhyb solution (Stratagene) with the indicated 3′ probe (Figure 1). Membranes were washed in 0.1xSSC, 0.2% SDS at 62°C twice, then once at 65°C. DNA samples from mice were prepared from tail clips and genotypes routinely determined by digestion of 5-10 μg of DNA with Bam HI and Eag I and analysis by hybridization as described above.

Blastocyst injection and mice breeding

ES cells of recombinant clones were injected into C57B1/6J blastocysts which were then transferred into pseudopregnant recipient female mice of strain C57BL/6J. Male chimaeric offspring were obtained with extensive ES cell contribution as judged by their agouti coat

colour. Five of these were bred with C57B1/6J female mice and produced agouti-coloured offspring indicating germline transmission. The genotype of these F1 mice was determined and TRB heterozygotes were crossed to generate litters containing homozygous mutants. All analyses were performed with progeny obtained from crosses between these TRB heterozygotes and thus represented hybrid mice derived from 129/sv (ES cell) and C57bl/6J strains.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis of mutant gene products

Total cellular RNA from selected tissues of wild type, heterozygous and homozygous mutant TRB mice was prepared and used to make first strand cDNA using as primer an antisense oligonuclcotide derived from the 3' terminal coding exon of the mouse TRβ gene. RT-PCR analysis was then performed on the cDNA using the pairs of primers indicated in Figure 2 that specifically amplify products representing the N-terminal coding regions of the two TRβ N-terminal variant proteins (TRβ1 and TRβ2) that are encoded by the TRβ gene. The products from mice of all three genotypes were purified and their DNA sequences were determined by automated sequencer.

RT-PCR analysis of products of the wild type and mutant alleles

RT-PCR products of RNA from different tissues from wild type (+/+), heterozygous (-/+) and homozygous mutant (-/-) mice were generated using pairs of primers that specifically amplify products derived from TRβ1 and TRβ2, as indicated in the lower part of the figure. Products were electrophoresed on 0.8% agarose gels and visualised by ethidium bromide staining. In all tissues from homozygous mutant mice, the RT-PCR products were 100 bp shorter than in

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Example 2

Analysis of the effect of the thyroid hormone receptor β on the development of auditory function

Mice which were heterozygous ($Thrb^{-/+}$) were prepared as described above. The auditory-evoked brainstem response (ABR) was tested in these mice. It was found that the threshold sound pressure levels required for ABR were significantly elevated ($p<<0.01$) for all pure tones tested (8,16 and 32 kHz) and for a click stimulus in all adult $Thrb^{-/-}$ mice. $Thrb^{-/+}$ and control $Thrb^{+/+}$ mice both had ABR thresholds in the normal range, whereas $Thrb^{-/-}$ mice displayed significantly elevated thresholds that were often in the 70-100 dB range, indicating severe impairment. Indeed, 10-15% of $Thrb^{-/-}$ mice were profoundly deaf since no response could be evoked with any frequency tested at 100 dB, the upper limit of the apparatus. In mutants in which a response could be evoked, albeit with elevated thresholds, the resultant ABR waveforms were not significantly different from those of the controls, with normal peaks and latencies, indicating that brainstem auditory functions were normal and suggesting a defect in the generation of the primary action potential from the cochlea. Since the impairment was general with respect to all frequencies tested, the defect was not restricted to particular regions of the cochlea that are responsive to specific frequencies. There was not evidence for vestibular defects, since $Thrb^{-/-}$ mice showed no circling or other abnormal behaviour. Analysis of mice at 2-3 weeks of age when hearing normally approaches adult sensitivity levels, also demonstrated impairment in $Thrb^{-/-}$ mice ($p<<0.01$) compared to controls. This confirmed that the mutation caused a permanent failure of development of auditory function.

Example 3

Physiological effects of targeted interaction of the mouse Trβ gene.

Thyroid pathology in homozygous mutants

$Thrb^{-/-}$ mice produced as described above were viable, they displayed normal growth rates and weight gain and they were fertile. Necropsy failed to reveal gross abnormalities in most organs, with the exception of the thyroid gland which was variably enlarged in $Thrb^{-/-}$ mice. Quantitative image analysis of histological sections indicated that thyroid areas were 1.5-2.0 fold increased ($P<0.05$) in overall size in homozygotes (mean ± SEM in mm^2, 0.58±0.09, n = 10) compared to heterozygous (0.35±0.04, n = 9) and wild type (0.39±0.04, n = 8) mice at 5 weeks of age. There was no significant difference between $Thrb^{-/+}$ and $Thrb^{+/+}$ mice. Higher magnificent revealed a diffuse enlargement of $Thrb^{-/-}$ thyroid glands resulting from an increase in both the numbers and size of follicles. The colloid of follicles from $Thrb^{-/-}$ mice frequently contained large phagocytic-like cells that were often multi-nucleated and other cellular debris that was probably derived from degenerating epithelial cells.

This pathology suggested that the $Thrb^{-/-}$ thyroid glands were in a hyperactive state with increased epithelial cell turnover, indicating that the mutation caused a recessive hyperthyroid-like condition. No difference was detected between the sexes and the enlargement persisted in mice analysed at 5, 18 and 40 weeks of age. The condition was not progressive since the pathology was not more pronounced, with no evidence of hyperplasia, in 40 week old mice. Image analysis of thyroid sections demonstrated an approximately constant ratio of areas of colloid:epithelium in $Thrb^{-/-}$ (mean ± SEM, 1.02±0.08, n - 10). $Thrb^{-/+}$ (0.86±0.1, n = 9) and

$Thrb^{+/+}$ (0.91±0.1, n - 8) mice. Thyroid size increased in all genotypes with age, but there was no significant difference in the ratio of colloid:epithelium between $Thrb^{-/-}$ and normal mice. The thyroid glands of $Thrb^{-/-}$ mice at postnatal day 7 also displayed an increase in the numbers and size of colloid-containing follicles indicating that the condition arose at an early age.

Hormonal disorder

The observed thyroid pathology of the $Thrb^{-/-}$ mice suggested that there could be abnormalities in thyroid hormone levels. Analysis of serum thyroid hormones revealed that the levels of total thyroxine (TT4), the major product of the thyroid gland, were significantly elevated in $Thrb^{-/-}$ mice at 5 - 40 weeks of age, irrespective of gender. Fig. 4A shows that mean TT4 levels were elevated ~2.5 fold in a representative analysis of 10 week old mice (means ±SEM for $Thrb^{-/-}$, $Thrb^{+/+}$ were 11.5±1.07, 4.6±0.3, 4.1±0.3 µg/dL, respectively). Parallel increases in free T4 were observed in $Thrb^{-/-}$ mice (1.7±0.18 ng/dL) compared to $Thrb^{+/+}$ (0.6±0.05) and $Thrb^{+/+}$ (0.5±0.06) mice. This confirmed the predicted thyroid hyperactivity and excluded abnormal serum binding or transport of T4 as the cause of the elevated serum hormone levels. Preliminary data indicated that there was a general decrease of TT4 levels in older $Thrb^{-/-}$ mice (~1.5 years of age), suggesting that the hyperactivity was ameliorated with age. The levels of total and free T3, the main biologically active form of thyroid hormone, were also elevated in $Thrb^{-/-}$ mice. The levels of total T3 were somewhat variable regardless of the genotype, perhaps indicating variability in the peripheral conversion of T4 to T3 in this mouse strain. However, free T3 levels were consistently elevated.

Failure to regulate thyroid stimulating hormone

Elevation of thyroid hormone levels normally suppresses TSH production by the pituitary thyrotropes. However, the mean serum levels of TSH were significantly elevated in $Thrb^{-/-}$ compared to $Thrb^{-/+}$ or $Thrb^{+/+}$ mice at 5-40 weeks of age, irrespective of gender. Thus, despite the high levels of thyroid hormones, TSH was paradoxically elevated in $Thrb^{-/-}$ mutants. Northern blot analysis of pituitary RNA showed that levels of mRNA encoding TSHα and TSHβ subunits were elevated 2.5 and 3.3-fold respectively compared to $Thrb^{+/+}$ mice, suggesting that the increased TSH levels in mice lacking Trβ reflected abnormal regulation of TSH gene transcription. Histological examination of pituitary glands from $Thrb^{-/-}$ mice revealed no abnormalities and immunohistochemical analysis showed no abnormal pattern of cells straining positively for the TSH subunits (Fig. 4D-G). Thus, the over-production of TSH detected in $Thrb^{-/-}$ mice resulted from defective thyrotrope function rather than from hyperplasia malformation of the pituitary gland.

Central nervous system (CNS) function and anatomy

The absence of, or excessive exposure to T3 during a critical embryonic and neonatal period can impair brain development (Legrand, 1984). To investigate if the absence of Trβ and/or the associated increase in thyroid hormone levels caused neurological defects, the function of the nervous system in $Thrb^{-/-}$ mice were assessed using a range of behavioural tests. These analyses were valid since mice, like humans or rats, are susceptible to behavioural defects associated with congenital thyroid disorders and similar tests have demonstrated learning disabilities in the hypothyroid (hyt) mutant mouse (Anthony *et al.*, 1993). In a stringent version of the Morris water task, requiring the mice to locate a hidden platform to escape. $Thrb^{-/-}$ and $Thrb^{+/+}$ mice learned to escape equally well with repeated trials over nine days.

When the platform was removed, mice of both genotypes spent equivalent time and activity in the quadrant where the platform had been located. Context fear conditioning and responses to paired stimuli that may indicate attention deficits were not significantly different in $Thrb^{-/-}$ mice (data not shown). However, these studies may not be conclusive as they employ an acoustic stimulus to which the mutants could not respond reliably due to defective auditory function (Forrest *et al*, submitted). In other tests such as activity in an open field and Y-maze, $Thrb^{-/-}$ and $Thrb^{+/+}$ mice also behaved similarly. Histological and histochemical analysis of the CNS of $Thrb^{-/-}$ mice revealed no obvious abnormalities in brain anatomy, including structures known to be sensitive to T3, such as the cerebellum hippocampus. Furthermore, analysis of hippocampal field potentials did not indicate defects in long term potentiation. In conclusion, while development delays and attention deficits were not excluded, no overt neurological defects were detected in adult $Thrb^{-/-}$ mutants, suggesting that Trβ has subtle rather than major functions in neurodevelopment.

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CLAIMS:

1. A transgenic mammal which is heterozygous or homozygous for an at least partially defective throid hormone receptor β gene.

2. A transgenic animal according to claim 1 in which the defective thyroid hormone receptor β gene has been produced by an insertion, deletion, substitution or inversion or other suitable genetic manipulation.

3. A transgenic animal according to claim 1 which is a rodent.

4. A transgenic animal according to claim 3 which is a mouse.

5. Cells derived from the transgenic mammal of claim 1 which are heterozygous or homozygous for defective throid hormone receptor β.

6. A method of producing a transgenic animal in accordance with claim 1, the method comprising the steps of:

   1) preparing a gene encoding an at least partially defective thyroid hormone receptor β as described above;

   2) introducing that thyroid hormone receptor β gene into suitable carrier cells;

   3) inserting those carrier cells into an embryo; and

4) replacing the embryo into a mother, and allowing the embryo to develop to full term.

7. A method of testing the agonist/antagonist properties of a compound in relation to a thyroid hormone receptor, the method comprising the steps of:
contacting a transgenic animal in accordance with claim 1 with the compound and monitoring the subsequent behavioural development of the animal.

8. A method of testing the agonist/antagonist properties of a compound in relation to a thyroid hormone receptor, the method comprising the steps of:
contacting cells in accordance with claim 5 with the compound and subsequently monitoring the cells.

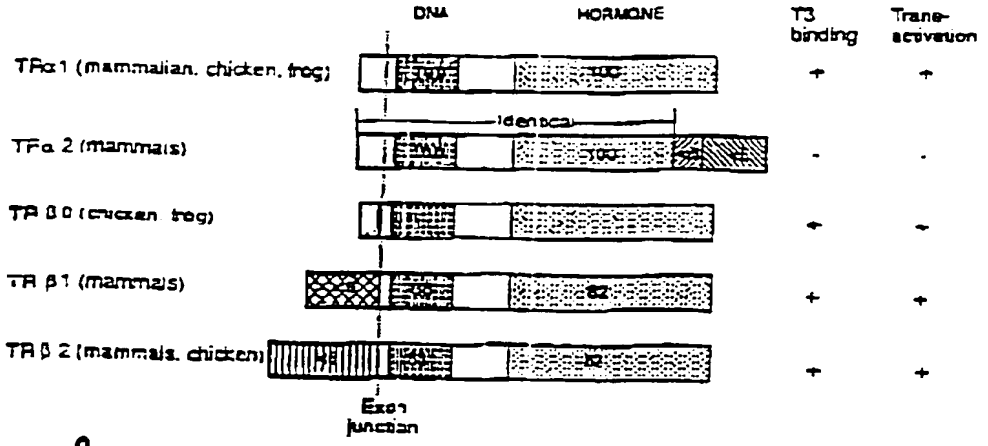


Figure A Schematic representation of thyroid hormone receptors in different species. The numbers indicate percent amino acid homology.

Fig B FRAGMENT OF THE GENOMIC TR CLONE

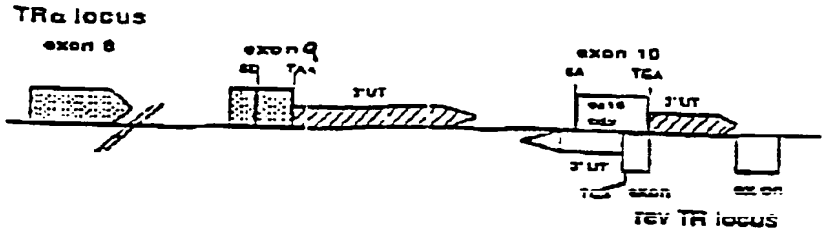
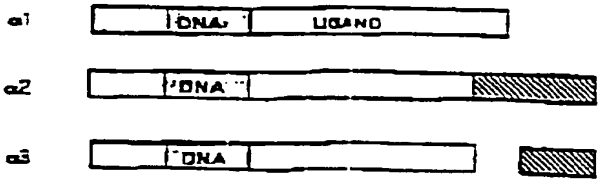


Fig C TRα VARIANTS



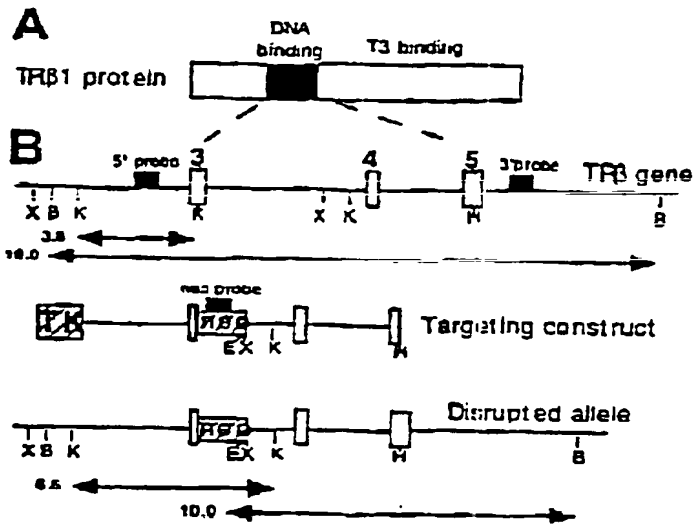


Figure 1
Disruption of the TRβ gene by homologous recombination

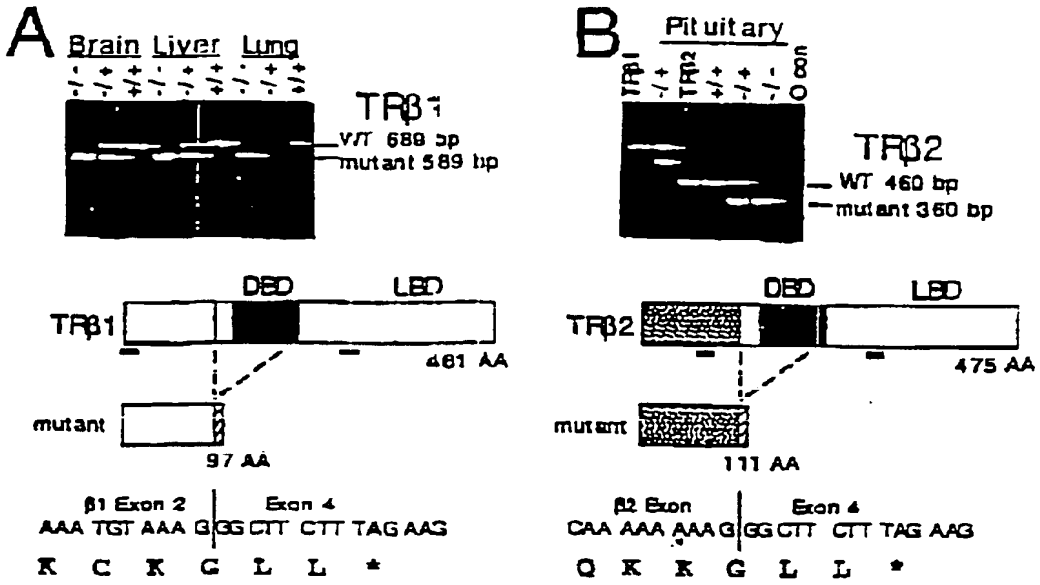


Figure 2

RT-PCR analysis of products of the wild type and mutant alleles.

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(71) Applicant *(for all designated States except US)*: KARO BIO AB [SE/SE]; Novum, S-141 57 Huddinge (SE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FORREST, Douglas [US/US]; 221 Harrison Street, Nutley, NJ 07110 (US). CURRAN, Thomas [US/US]; 15 Glenridge Parkway, Bloomfield, NJ 07003 (US).

(74) Agent: DEAN, John, Paul; Withers & Rogers, 4 Dyer's Buildings, Holborn, London EC1N 2JT (GB).

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(57) Abstract

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Inter[illegible]onal Application No
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IPC 6 C12N15/12 A01K67/027 C07K14/72 C12N5/10 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.

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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Alt, G

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	MOLECULAR MEDICINE, vol. 1, no. 3, March 1995, pages 309-319, XP000603622 WONG, R. ET AL.: "Cell type-dependent modulation of the dominant negative action of human mutant thyroid hormone beta1 receptors" see page 309, right-hand column to page 312, left-hand column ---	5
X	MOLECULAR ENDOCRINOLOGY, vol. 4, no. 5, May 1990, pages 715-720, XP000603789 O'DONNELL, A. AND KOENIG, R.: "Mutational analysis identifies a new functional domain of the thyroid hormone receptor" see page 719, left-hand column, "Transfections" ---	5
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INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/EP 96/01983

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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P,X	THE EMBO JOURNAL, vol. 15, no. 12, 17 June 1996, pages 3006-3015, XP000604851 FORREST, D. ET AL.: "Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor beta: evidence for tissue-specific modulation of receptor function" see the whole document -----	1-6

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. onal Application No
PCT/EP 96/01983

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		EP-A-	0692026	17-01-96
		JP-T-	8506246	09-07-96

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TRANSGENIC ANIMALS HAVING A DEFECTIVE THYROID HORMONE RECEPTOR BETA GENE

This application relates to transgenic animals, particularly mice, and tissues and cell lines thereof that in a homozygous form lack the gene for thyroid hormone receptor β (TRβ). The mice, tissues and cell lines of the invention may be used in the testing for pharmaceutical or clinical purposes of substances such as thyroid hormones T_3 and T_4 and possible antagonists and agonists thereof.

The thyroid hormones tri-iodothyronine (T_3) and thyroxine (T_4) have a very wide range of effects. In adult mammals they influence nearly all organs, the metabolism of nutrients, basal metabolic rate and oxygen consumption. In humans, the deficiency or excess of circulating thyroid hormones results in the well characterised syndromes hypo- and hyperthyroidism.

The thyroid hormones are essential for the normal development of the central nervous system particularly in the foetal and neonatal stages [1-6]. Deficiencies in the action of thyroid hormones lead to hypothyroidism that can be due to either acquired or congenital disorders. Some of the congenital causes of hypothyroidism are embryopathies as absence, hypoplasia, or ectopic localization of the thyroid gland; enzymatic disorders; deficient hormone synthesis and receptor disorders (Generalized Thyroid Hormone Syndrome (GRTS)). Unless treated, congenital hypothyroidism leads to irreversible mental retardation and short stature (dwarfism). Other symptoms include neurological dysfunctions such as poor coordination and balance, abnormal fine motor movements, speech problems, spasticity, tremor and hyperactive

deep tendon reflexes. In addition basal metabolic rate, gluconeogenesis, lipogenesis and cardiac output are decreased. Hypothyroidism in adults leads to symptoms similar to those described above, except for the mental retardation. However, adult patients are easily treated with hormone therapy.

In contrast to congenital hypothyroidism, hyperthyroidism is more common in adults. In general, the symptoms are the reverse: increased metabolism, lower serum cholesterol levels, hyperactivity and tachycardia are hallmarks of elevated T3/T4 levels [25].

Thyroid hormones act through thyroid hormone receptors (TRs) which belong to the superfamily of steroid hormone receptors. TRs are ligand dependent transcription factors which regulate the transcription of their target genes through responsive elements in the DNA. In vertebrates there are a variety of TRs[7-13] (Fig. A) derived from TRα and TRβ genes, which are located at the 17th and 3rd chromosomes respectively in humans. There is considerable homology between the TRα and TRβ proteins and between the receptors in different species, such as rat, mouse, and human. The α-gene encodes the subtypes α1 and α2. The α2 subtype is not a functional receptor in the sense that it lacks T_3/T_4 hormone binding capability. The β-gene encodes the subtypes β1 and β2. The latter has so far been identified only at the messenger RNA level. The physiological significance of these different proteins has not yet been clarified. Different amino- and carboxy-termini for the TR variants suggest different trans-activating properties for TRα and Trβ. In addition, the differential expression during brain development suggest different roles for the TR variants during development [14-16].

The mechanism of T_3 action via its receptor is quite complex due to the presence of multiple TRs [17-20]. The TRα locus encodes in addition to the TRα gene another receptor denoted as Rev-α. Rev-α arises by transcription of the opposite strand of TRα gene and overlaps the α2 region at the 3' end (Fig. B). Furthermore, there are TRα2 and TRα3 variants; the protein sequence of the latter is identical to that of TRα2 with the exception that it lacks the first 42 amino acids of the carboxy terminus (Fig. C).

In humans, the GRTS has been related to TRβ gene disorders. No clinical syndromes have yet been associated to TRα gene mutations suggesting that the TRα gene is either dispensable or essential for life. It is equally unclear as to which of the two thyroid hormone receptors the actions of thyroid hormones can be ascribed in hypo- and hyperthyroidism. If the individual functions in hormone action of the receptors could be identified, agonists or agonists that are specific for either of the receptors could be used for treatment of specific target tissues without adversely affecting other tissues.

Treatment of many diseases associated with thyroid hormone function cannot be done today since administration of increased doses of the hormone to achieve a desired effect in a given tissue, leads to adverse effects in another. The effects of thyroid hormones are mediated by two different receptors that are coexpressed in some tissues, whereas other tissues express only one of them. It should therefore be possible to design agonists and antagonists that are specific for each of the receptors and that can mediate a desired activation or repression of receptor function.

In order to allow testing of such components we have disrupted the TRβ gene in the mouse genome, and bred such animals to homozygosity. These animals can grow to at least sexual maturity, and are therefore suitable tools for identifying the action of agonists and antagonists of TRβ.

According to one aspect of the invention there is provided a transgenic mammal which is heterozygous for an at least partially defective thyroid hormone receptor β gene. The defective gene may be inactivated for example by an insertion, deletion, substitution or inversion or any other suitable genetic manipulation.

Preferably, the mammal is a rodent, more preferably a mouse.

One heterozygous transgenic mammal in accordance with the invention may be bred with another such heterozygous transgenic mammal to produce a mammal which is homozygous for a defective thyroid hormone receptor β gene. Thus according to another aspect of the invention there is provided a transgenic mammal which is homozygous for an at least partially defective β thyroid hormone receptor β gene.

The invention also provides cells derived from the animal of the invention which are heterozygous or homozygous for a defective thyroid hormone receptor β.

According to another aspect of the invention there is provided a method of producing a transgenic animal in accordance with the invention the method comprising :

1) preparing a gene encoding an at least partially defective thyroid hormone receptor β as described above;

2) introducing that gene into suitable carrier cells;

3) inserting those carrier cells into an embryo; and

4) replacing the embryo into a mother, and allowing the embryo to develop to full term.

According to a further aspect of the invention there is provided a method of testing the agonist/antagonist properties of a compound in relation to the thyroid hormone receptor, the method comprising:

contacting a transgenic animal in accordance with the invention with the compound and monitoring subsequent development of the animal.

Alternatively, the method may involve using cells or tissues derived from the transgenic animal.

The transgenic mammal of the invention is suitable for testing the effects of agonists and antagonists of thyroid hormone action, in particular those that discriminate between TRα and TRβ. In particular, the transgenic mammal of the invention or cells or tissues derived therefrom can be used to study the following:

1. Administration of excess thyroid hormones decreases high serum cholesterol levels. However, an adverse side effect is that cardiac output also increases which can lead to arrythmia. If these two functions of thyroid hormones are mediated by distinct

receptors, a proper administration of receptor specific agonists or antagonists would lead to the desired decrease in serum cholesterol while leaving cardiac function normal.

2. Hypo- and hyperthyroidism adversely affect bone structure. The use of receptor-specific thyroid hormone antagonists or agonists for treatment of e.g hypercholesterolemia or other diseases must therefore include a test for their influence on bone synthesis and turnover.

3. Regulation of heart functions such as pulse, arrythmia, or myocardiac muscle can be targeted by the use of receptor specific thyroid hormone antagonists or agonists.

4. Many organs or tissues produce hormones in a thyroid hormone dependent manner. Such tissues include the hypophysis (producing growth hormone, prolactin, thyroid stimulating hormone, luteinizing hormone), the hypothalamus (thyrotropin releasing hormone, oxytocin), peripheral tissues (insulin growth factor I). The effect of receptor specific thyroid hormone antagonists or agonists on such endocrine systems can be determined with the mammals of the present invention.

5. Basal metabolic rate, gluconeogenesis, lipogenesis, lipolysis and thermogenesis are increased in hyperthyroidism and decreased during hypothyroidism. The effect of receptor specific thyroid hormone antagonists or agonists on such metabolic processes can be determined with the mammal of the present invention.

6. Toxic effects of agonists and antagonists on normal and abnormal physiological metabolic processes.

7. Effects on brain or other neuronal function (hearing, peripheral nervous system), as well as effects on embryonal and foetal development of receptor specific thyroid hormone antagonists or agonists on such endocrine systems can be determined with the transgenic mammal of the present invention.

8. Effects on increasing or decreasing body growth in patients with growth disorders.

9. A large number of genes or gene products are known to be regulated by thyroid hormones. The effects of agonists and antagonists of such systems before clinical trials can commence.

10. Effect on haemopoiesis. Hypothyroid patients are usually anaemic.

11. Treatment of patients that have defective TRα receptor genes. As mentioned above, no patients with mutant TRα genes have been found, whereas genetic defects in more than 250 patients with defective TRβ genes have been identified. The latter patients were first clinically identified due to their inappropriate levels of thyroid hormones and other thyroid hormone regulated hormones such as TSH. It is therefore possible that diseases due to defects in the TRα gene have remained undetected because the patients have normal T_3/T_4 and TSH levels and their symptoms therefore would not

be easily associated with a receptor dysfunction. The TRβ deficient mammals of the present invention allow the identification of such disease, symptoms, and their cure with suitable agonists.

Mammals in accordance with the invention and their production will now be described by way of example only with reference to the further accompanying drawings Figures 1-2 in which:

Fig. 1 illustrates disruption of the TRβ gene by homologous recombination; and

Fig. 2 illustrates an RT-PCR analysis of products of the wild type and mutant alleles of the TRβ gene.

Example 1

Generation of mutant mouse with deleted thyroid hormone receptor β gene.

EXPERIMENTAL PROCEDURES

Targeting vector

A chick TRβ cDNA insert was used to screen a bacteriophage lambda library of genomic DNA of a 129sv strain mouse (Stratagene) to obtain overlapping clones that encompassed the entire coding domain of the TRβ gene. Fig. 1A is a schematic representation of the TRβ1 protein showing the central DNA binding domain (filled in black) and C-terminal T3-binding domain. Fig. 1B top line, illustrates the structure of the central region of the gene containing the first three coding exons that are common for both TRβ1 and TRβ2 (here numbered 3 to

5). The middle line illustrates the targeting vector contained 3 kbp and 4 kbp respectively of 5′ and 3′ homologous flanking DNA and carried a 3 kbp deletion including part of exon number 3. The bottom line shows the structure of the mutant allele generated by homologous recombination 5′, nco and 3′ probes used in Southern blot analyses are shown as well as the band sizes predicted to be detected with the 3′ probe following digestion with Barn HI and Eag I: the wild type band size being 19 kbp whereas the mutant band is 10 kbp. Restriction enzyme sites are indicated where relevant. X, Xba I; B, Bam HI; K, Kpn I; E, Eag I. The exon structure was confirmed by DNA sequencing of plasmid sub-clones. The targeting vector (Figure 1B) contained from 5′ to 3′: a TK gene fragment from pMCI-HSV TK, a 3 kbp fragment of TRβ genomic DNA extending to a Kpn I site in the coding exon number 3, a neomycin resistance gene from pgkneobpA, a 4 kbp Xba-I-Hind III genomic fragment containing the TRβ exons 4 and 5. The construct was linearized at the 5′ end of the TK gene by Bam HI digestion prior to electroporation.

Electroporation and selection of ES cells

W9.5 male ES cells derived from 129/sv mice were grown on feeder layers of G418-resistant primary mouse embryo fibroblasts (PMEFs) in dishes that had been treated with 0.1% gelatin: PMEFs were mitotically inactivated by gamma-irradiation. W9.5 cells were cultured in Dulbecco's Modified Eagle medium (Specialty Media) supplemented with 15% defined fetal bovine serum (Hyclone), 1000 U/ml of recombinant LIF (Gibco), L-glutamine, non-essential amino acids, β-mercaptoethanol and antibodies as described [26] 3 x 10^7 W9.5 cells at passage 12 were resuspended in 0.8 ml PBS containing 25μg of linearized targeting vector DNA for electroporation using a Bio-Rad Gene Pulser (500μF, 250V). Cells were then plated onto

60mm dishes. The next day the medium was replaced with medium containing 350 μg/ml G418 (dry weight, Gibco) and on day two, 2μM ganciclovir (a gift of Syntex Corp. Palo Alto, CA) was added. The medium was replaced each day and on day 8, colonies were picked and transferred into 48 well dishes. After 4-5 days growth in 48-well plates, each clone was trypsinized and 9/10 of the suspension volume removed for DNA preparation. To the remaining volume, fresh medium and PMEFs were added. Clones identified as positive for homologous recombination were expanded and stocks frozen. The chromosome content of positive clones was determined by growth on microscope chamber slides for analysis *in situ*.

Southern blot hybridization analysis of ES cell clones and genotype determination

ES cells colonies were screened for homologous recombinants in pools of six. Cell pellets were lysed at 55°C overnight and DNA was prepared and digested overnight with Bam HI and Eag I, then analyzed on 0.7% agarose gels. DNA was transferred to Duralose-UV membrane and hybridized using Quickhyb solution (Stratagene) with the indicated 3′ probe (Figure 1). Membranes were washed in 0.1xSSC, 0.2% SDS at 62°C twice, then once at 65°C. DNA samples from mice were prepared from tail clips and genotypes routinely determined by digestion of 5-10 μg of DNA with Bam HI and Eag I and analysis by hybridization as described above.

Blastocyst injection and mice breeding

ES cells of recombinant clones were injected into C57B1/6J blastocysts which were then transferred into pseudopregnant recipient female mice of strain C57BL/6J. Male chimaeric offspring were obtained with extensive ES cell contribution as judged by their agouti coat

colour. Five of these were bred with C57B1/6J female mice and produced agouti-coloured offspring indicating germline transmission. The genotype of these F1 mice was determined and TRB heterozygotes were crossed to generate litters containing homozygous mutants. All analyses were performed with progeny obtained from crosses between these TRB heterozygotes and thus represented hybrid mice derived from 129/sv (ES cell) and C57bl/6J strains.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis of mutant gene products

Total cellular RNA from selected tissues of wild type, heterozygous and homozygous mutant TRB mice was prepared and used to make first strand cDNA using as primer an antisense oligonucleotide derived from the 3' terminal coding exon of the mouse TRβ gene. RT-PCR analysis was then performed on the cDNA using the pairs of primers indicated in Figure 2 that specifically amplify products representing the N-terminal coding regions of the two TRβ N-terminal variant proteins (TRβ1 and TRβ2) that are encoded by the TRβ gene. The products from mice of all three genotypes were purified and their DNA sequences were determined by automated sequencer.

RT-PCR analysis of products of the wild type and mutant alleles

RT-PCR products of RNA from different tissues from wild type (+/+), heterozygous (-/+) and homozygous mutant (-/-) mice were generated using pairs of primers that specifically amplify products derived from TRβ1 and TRβ2, as indicated in the lower part of the figure. Products were electrophoresed on 0.8% agarose gels and visualised by ethidium bromide staining. In all tissues from homozygous mutant mice, the RT-PCR products were 100 bp shorter than in

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Example 2

Analysis of the effect of the thyroid hormone receptor β on the development of auditory function

Mice which were heterozygous ($Thrb^{-/+}$) were prepared as described above. The auditory-evoked brainstem response (ABR) was tested in these mice. It was found that the threshold sound pressure levels required for ABR were significantly elevated ($p<<0.01$) for all pure tones tested (8,16 and 32 kHz) and for a click stimulus in all adult $Thrb^{-/-}$ mice. $Thrb^{-/+}$ and control $Thrb^{+/+}$ mice both had ABR thresholds in the normal range, whereas $Thrb^{-/-}$ mice displayed significantly elevated thresholds that were often in the 70-100 dB range, indicating severe impairment. Indeed, 10-15% of $Thrb^{-/-}$ mice were profoundly deaf since no response could be evoked with any frequency tested at 100 dB, the upper limit of the apparatus. In mutants in which a response could be evoked, albeit with elevated thresholds, the resultant ABR waveforms were not significantly different from those of the controls, with normal peaks and latencies, indicating that brainstem auditory functions were normal and suggesting a defect in the generation of the primary action potential from the cochlea. Since the impairment was general with respect to all frequencies tested, the defect was not restricted to particular regions of the cochlea that are responsive to specific frequencies. There was not evidence for vestibular defects, since $Thrb^{-/-}$ mice showed no circling or other abnormal behaviour. Analysis of mice at 2-3 weeks of age when hearing normally approaches adult sensitivity levels, also demonstrated impairment in $Thrb^{-/-}$ mice ($p<<0.01$) compared to controls. This confirmed that the mutation caused a permanent failure of development of auditory function.

Example 3

Physiological effects of targeted interaction of the mouse Trβ gene.

Thyroid pathology in homozygous mutants

$Thrb^{-/-}$ mice produced as described above were viable, they displayed normal growth rates and weight gain and they were fertile. Necropsy failed to reveal gross abnormalities in most organs, with the exception of the thyroid gland which was variably enlarged in $Thrb^{-/-}$ mice. Quantitative image analysis of histological sections indicated that thyroid areas were 1.5-2.0 fold increased ($P<0.05$) in overall size in homozygotes (mean ± SEM in mm^2, 0.58±0.09, n = 10) compared to heterozygous (0.35±0.04, n = 9) and wild type (0.39±0.04, n = 8) mice at 5 weeks of age. There was no significant difference between $Thrb^{-/+}$ and $Thrb^{+/+}$ mice. Higher magnificent revealed a diffuse enlargement of $Thrb^{-/-}$ thyroid glands resulting from an increase in both the numbers and size of follicles. The colloid of follicles from $Thrb^{-/-}$ mice frequently contained large phagocytic-like cells that were often multi-nucleated and other cellular debris that was probably derived from degenerating epithelial cells.

This pathology suggested that the $Thrb^{-/-}$ thyroid glands were in a hyperactive state with increased epithelial cell turnover, indicating that the mutation caused a recessive hyperthyroid-like condition. No difference was detected between the sexes and the enlargement persisted in mice analysed at 5, 18 and 40 weeks of age. The condition was not progressive since the pathology was not more pronounced, with no evidence of hyperplasia, in 40 week old mice. Image analysis of thyroid sections demonstrated an approximately constant ratio of areas of colloid:epithelium in $Thrb^{-/-}$ (mean ± SEM, 1.02±0.08, n - 10). $Thrb^{-/+}$ (0.86±0.1, n = 9) and

$Thrb^{+/+}$ (0.91±0.1, n - 8) mice. Thyroid size increased in all genotypes with age, but there was no significant difference in the ratio of colloid:epithelium between $Thrb^{-/-}$ and normal mice. The thyroid glands of $Thrb^{-/-}$ mice at postnatal day 7 also displayed an increase in the numbers and size of colloid-containing follicles indicating that the condition arose at an early age.

Hormonal disorder

The observed thyroid pathology of the $Thrb^{-/-}$ mice suggested that there could be abnormalities in thyroid hormone levels. Analysis of serum thyroid hormones revealed that the levels of total thyroxine (TT4), the major product of the thyroid gland, were significantly elevated in $Thrb^{-/-}$ mice at 5 - 40 weeks of age, irrespective of gender. Fig. 4A shows that mean TT4 levels were elevated ~2.5 fold in a representative analysis of 10 week old mice (means ±SEM for $Thrb^{-/-}$, $Thrb^{-/+}$ were 11.5±1.07, 4.6±0.3, 4.1±0.3 µg/dL, respectively). Parallel increases in free T4 were observed in $Thrb^{-/-}$ mice (1.7±0.18 ng/dL) compared to $Thrb^{-/+}$ (0.6±0.05) and $Thrb^{+/+}$ (0.5±0.06) mice. This confirmed the predicted thyroid hyperactivity and excluded abnormal serum binding or transport of T4 as the cause of the elevated serum hormone levels. Preliminary data indicated that there was a general decrease of TT4 levels in older $Thrb^{-/-}$ mice (~1.5 years of age), suggesting that the hyperactivity was ameliorated with age. The levels of total and free T3, the main biologically active form of thyroid hormone, were also elevated in $Thrb^{-/-}$ mice. The levels of total T3 were somewhat variable regardless of the genotype, perhaps indicating variability in the peripheral conversion of T4 to T3 in this mouse strain. However, free T3 levels were consistently elevated.

Failure to regulate thyroid stimulating hormone

Elevation of thyroid hormone levels normally suppresses TSH production by the pituitary thyrotropes. However, the mean serum levels of TSH were significantly elevated in $Thrb^{-/-}$ compared to $Thrb^{-/+}$ or $Thrb^{+/+}$ mice at 5-40 weeks of age, irrespective of gender. Thus, despite the high levels of thyroid hormones, TSH was paradoxically elevated in $Thrb^{-/-}$ mutants. Northern blot analysis of pituitary RNA showed that levels of mRNA encoding TSHα and TSHβ subunits were elevated 2.5 and 3.3-fold respectively compared to $Thrb^{+/+}$ mice, suggesting that the increased TSH levels in mice lacking Trβ reflected abnormal regulation of TSH gene transcription. Histological examination of pituitary glands from $Thrb^{-/-}$ mice revealed no abnormalities and immunohistochemical analysis showed no abnormal pattern of cells straining positively for the TSH subunits (Fig. 4D-G). Thus, the over-production of TSH detected in $Thrb^{-/-}$ mice resulted from defective thyrotrope function rather than from hyperplasia malformation of the pituitary gland.

Central nervous system (CNS) function and anatomy

The absence of, or excessive exposure to T3 during a critical embryonic and neonatal period can impair brain development (Legrand, 1984). To investigate if the absence of Trβ and/or the associated increase in thyroid hormone levels caused neurological defects, the function of the nervous system in $Thrb^{-/-}$ mice were assessed using a range of behavioural tests. These analyses were valid since mice, like humans or rats, are susceptible to behavioural defects associated with congenital thyroid disorders and similar tests have demonstrated learning disabilities in the hypothyroid (hyt) mutant mouse (Anthony *et al.*, 1993). In a stringent version of the Morris water task, requiring the mice to locate a hidden platform to escape. $Thrb^{-/-}$ and $Thrb^{+/+}$ mice learned to escape equally well with repeated trials over nine days.

When the platform was removed, mice of both genotypes spent equivalent time and activity in the quadrant where the platform had been located. Context fear conditioning and responses to paired stimuli that may indicate attention deficits were not significantly different in $Thrb^{-/-}$ mice (data not shown). However, these studies may not be conclusive as they employ an acoustic stimulus to which the mutants could not respond reliably due to defective auditory function (Forrest *et al*, submitted). In other tests such as activity in an open field and Y-maze, $Thrb^{-/-}$ and $Thrb^{+/+}$ mice also behaved similarly. Histological and histochemical analysis of the CNS of $Thrb^{-/-}$ mice revealed no obvious abnormalities in brain anatomy, including structures known to be sensitive to T3, such as the cerebellum hippocampus. Furthermore, analysis of hippocampal field potentials did not indicate defects in long term potentiation. In conclusion, while development delays and attention deficits were not excluded, no overt neurological defects were detected in adult $Thrb^{-/-}$ mutants, suggesting that Trβ has subtle rather than major functions in neurodevelopment.

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CLAIMS:

1. A transgenic mammal which is heterozygous or homozygous for an at least partially defective throid hormone receptor β gene.

2. A transgenic animal according to claim 1 in which the defective thyroid hormone receptor β gene has been produced by an insertion, deletion, substitution or inversion or other suitable genetic manipulation.

3. A transgenic animal according to claim 1 which is a rodent.

4. A transgenic animal according to claim 3 which is a mouse.

5. Cells derived from the transgenic mammal of claim 1 which are heterozygous or homozygous for defective throid hormone receptor β.

6. A method of producing a transgenic animal in accordance with claim 1, the method comprising the steps of:

   1) preparing a gene encoding an at least partially defective thyroid hormone receptor β as described above;

   2) introducing that thyroid hormone receptor β gene into suitable carrier cells;

   3) inserting those carrier cells into an embryo; and

4) replacing the embryo into a mother, and allowing the embryo to develop to full term.

7. A method of testing the agonist/antagonist properties of a compound in relation to a thyroid hormone receptor, the method comprising the steps of:
contacting a transgenic animal in accordance with claim 1 with the compound and monitoring the subsequent behavioural development of the animal.

8. A method of testing the agonist/antagonist properties of a compound in relation to a thyroid hormone receptor, the method comprising the steps of:
contacting cells in accordance with claim 5 with the compound and subsequently monitoring the cells.

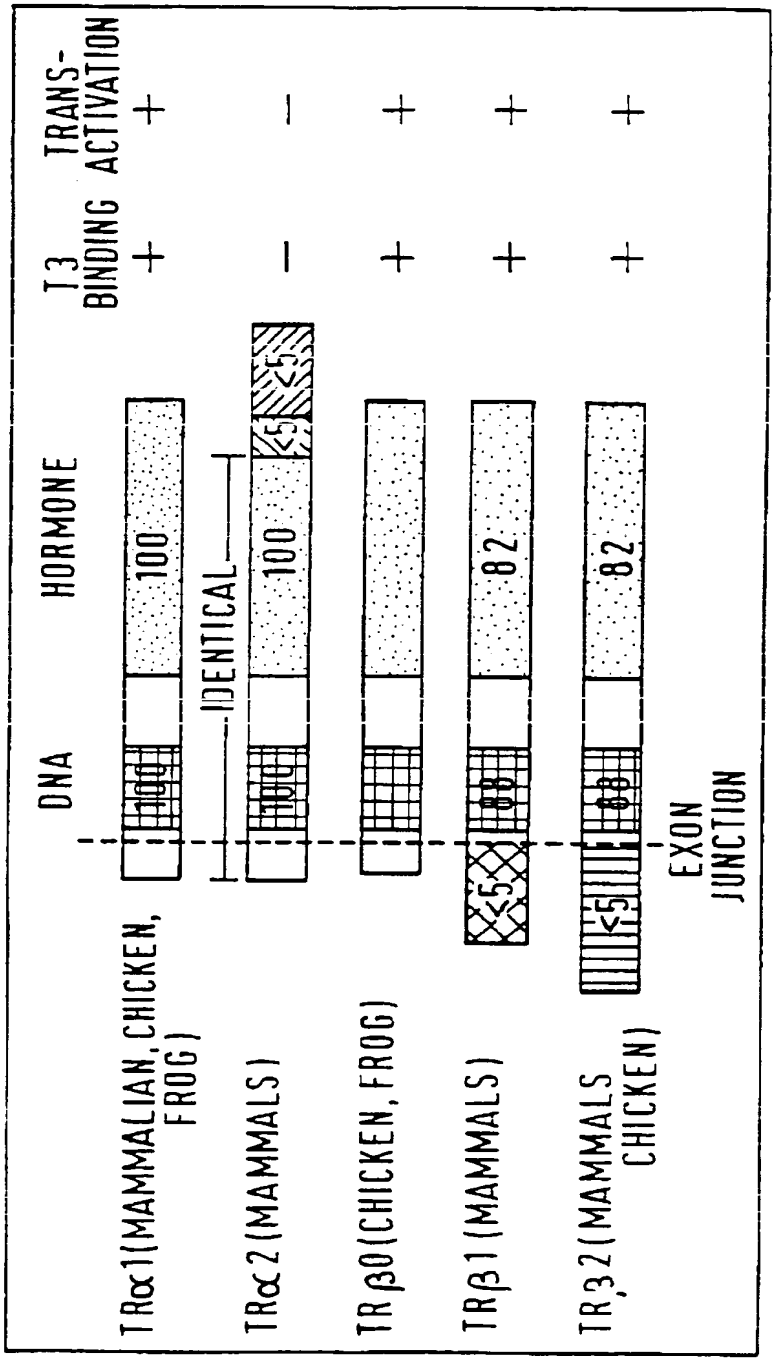


FIG. 1A

SCEMATIC REPRESENTATION OF THYROID HORMONE RECEPTORS IN DIFFERENT SPECIES. THE NUMBERS INDICATE PER CENT AMINO ACID HOMOLOGY.

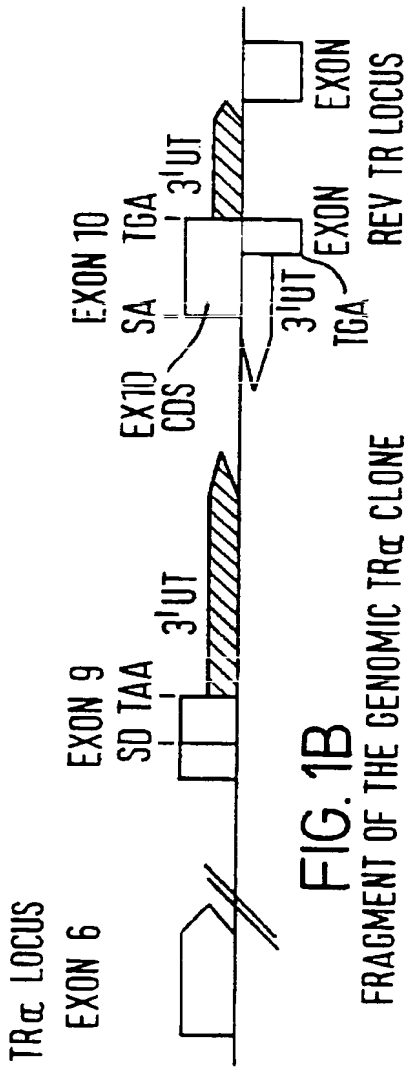


FIG. 1B
FRAGMENT OF THE GENOMIC TRα CLONE

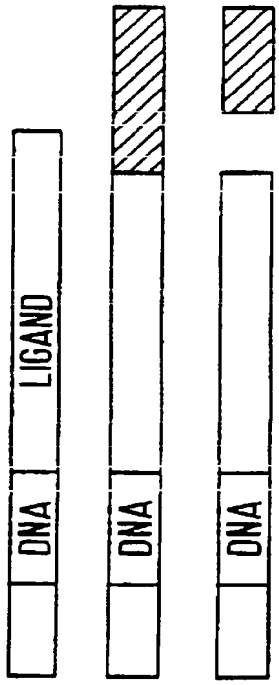


FIG. 1C
TRα VARIANTS

FIG. 2A

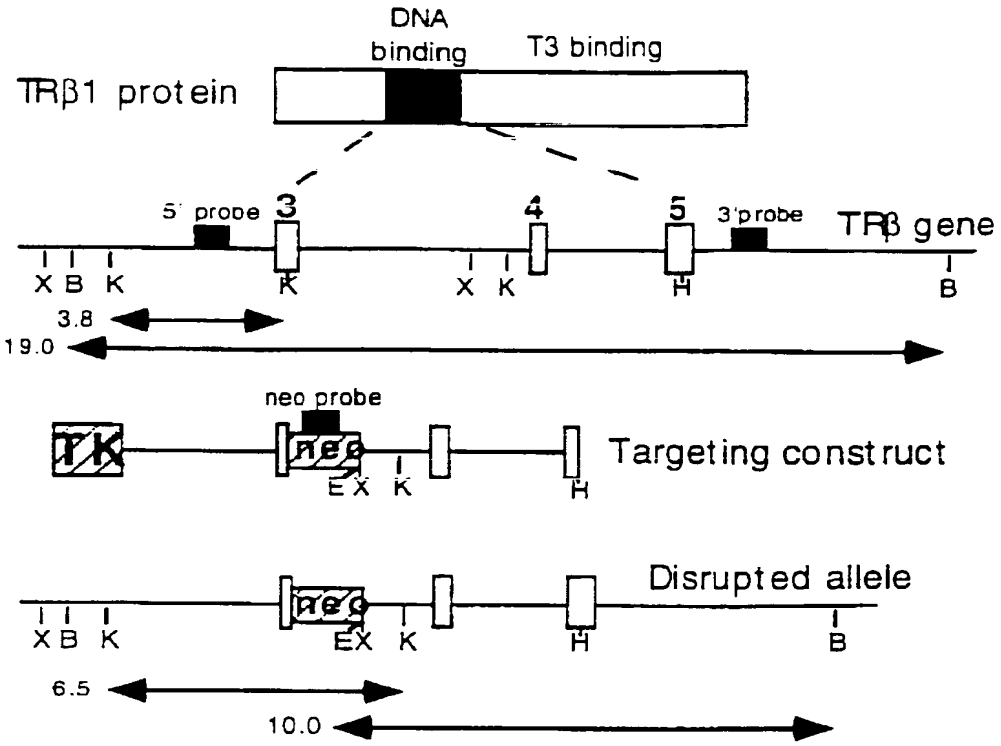

DNA
binding
T3 binding
TRβ1 protein
5' probe
3
4
5
3'probe
TRβ gene
X B K
K
X K
H
B
3.8
19.0
neo probe
TK
neo
E X K
H
Targeting construct
Disrupted allele
X B K
neo
E X K
H
B
6.5
10.0

FIG. 2B

FIG. 3A

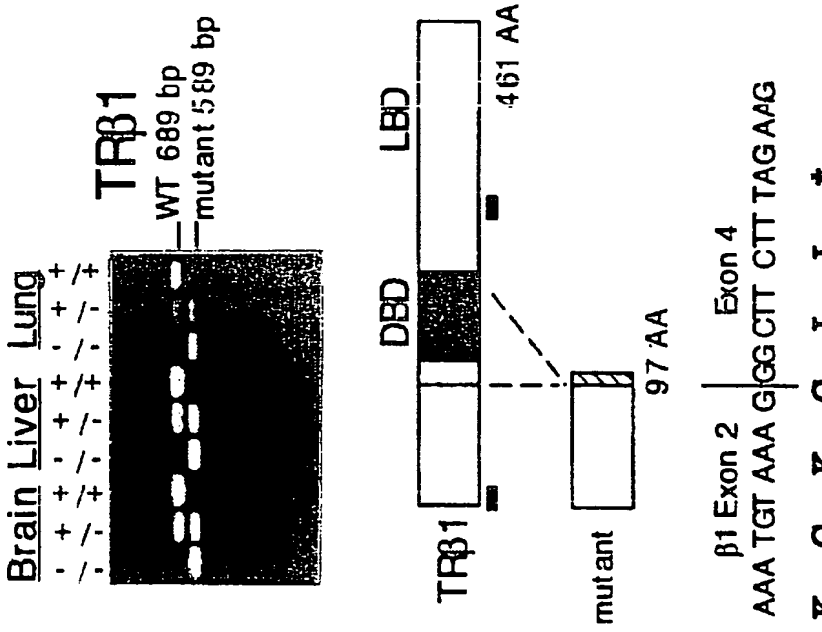
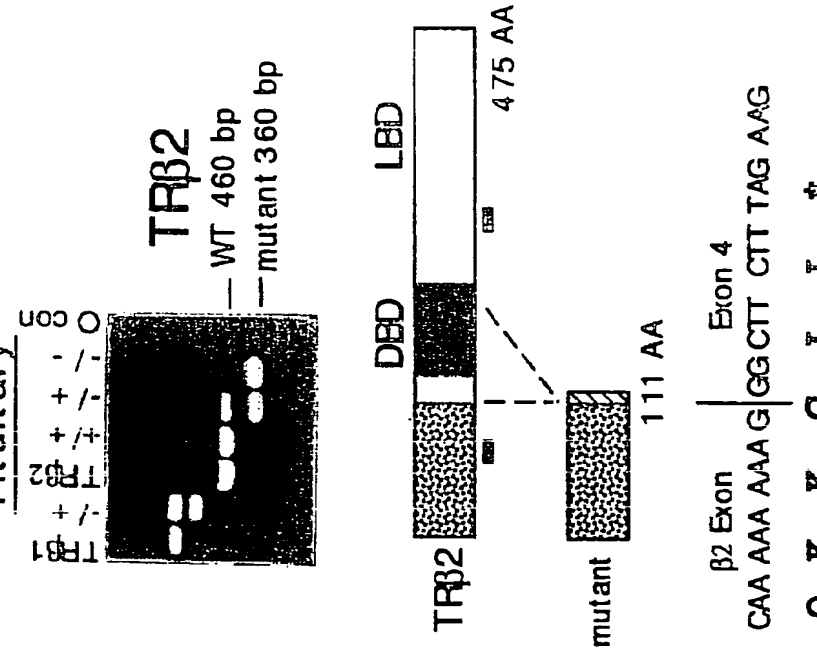

Brain Liver Lung
+/+
+/-
-/-
+/+
+/-
-/-
+/+
+/-
-/-
TRβ1
WT 689 bp
mutant 589 bp
DBD
LBD
461 AA
TRβ1
97 AA
mutant
β1 Exon 2
Exon 4
AAA TGT AAA G GG CTT CTT TAG AAG
K C K G L L *

FIG. 3B


Pituitary
con
-/-
+/-
+/+
TRβ2
+/-
+/-
TRβ1
TRβ2
WT 460 bp
mutant 360 bp
DBD
LBD
475 AA
TRβ2
111 AA
mutant
β2 Exon
Exon 4
CAA AAA AAA G GG CTT CTT TAG AAG
Q K K G L L *

INTERNATIONAL SEARCH REPORT

Inter ...nal Application No
PCT/EP 96/01983

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 A01K67/027 C07K14/72 C12N5/10 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	CLINICAL RESEARCH MEETING, SAN DIEGO, CALIFORNIA, MAY 5-8, 1995, XP002015493 WONG, R. ET AL.: "A transgenic model of resistance to thyroid hormone (RTH): Correlation of mutant thyroid hormone beta receptor (TRbeta1) levels with the phenotype of fat loss and hyperactivity"	1-4,6
X	& JOURNAL OF INVESTIGATIVE MEDECINE, vol. 43, no. Suppl.2, April 1995, page 223A WONG, R. ET AL.: "A transgenic model of resistance to thyroid hormone (RTH): correlation of mutant thyroid hormone beta (TRbeta1) levels with the phenotype of fat loss and hyperactivity" see the whole document	1-4,6
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
9 October 1996	31. 10. 96

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

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PCT/EP 96/01983

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CLINICAL INVESTIGATION, vol. 88, December 1991, pages 2123-2130, XP000574480 PARILLA, R. ET AL.: "Characterization of seven novel mutations of the c-erbAbeta gene in unrelated kindreds with generalized thyroid hormone resistance" see page 2124, left-hand column, first paragraph ---	1,2
X	MOLECULAR MEDICINE, vol. 1, no. 3, March 1995, pages 309-319, XP000603622 WONG, R. ET AL.: "Cell type-dependent modulation of the dominant negative action of human mutant thyroid hormone beta1 receptors" see page 309, right-hand column to page 312, left-hand column ---	5
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A	WO,A,94 24282 (PFIZER INC.) 27 October 1994 see page 13 ---	7,8
A	MOLECULAR ENDOCRINOLOGY, vol. 8, no. 12, December 1994, pages 1605-1617, XP000603682 WOOD, W.M. ET AL.: "Structural and functional characterization of the genomic locus encoding the murine beta2 thyroid hormone receptor" see Figure 2 ---	1
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INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/EP 96/01983

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MOLECULAR ENDOCRINOLOGY, vol. 10, no. 1, 1996, pages 100-106, XP000603520 HAYASHI, Y. ET AL.: "A mouse model of resistance to thyroid hormone produced by somatic gene transfer of a mutant thyroid hormone receptor" see the whole document ---	1-6
P,X	THE EMBO JOURNAL, vol. 15, no. 12, 17 June 1996, pages 3006-3015, XP000604851 FORREST, D. ET AL.: "Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor beta: evidence for tissue-specific modulation of receptor function" see the whole document -----	1-6

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. onal Application No
PCT/EP 96/01983

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9424282	27-10-94	CA-A-	2159813	27-10-94
		EP-A-	0692026	17-01-96
		JP-T-	8506246	09-07-96